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# Recent advances in the development of synthetic chemical probes for glycosidase enzymes

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The emergence of synthetic glycoconjugates as chemical probes for the detection of glycosidase enzymes has resulted in the development of a range of useful chemical tools with applications in glycobiology, biotechnology, medical and industrial research. Critical to the function of these probes is the preparation of substrates containing a glycosidic linkage that when activated by a specific enzyme or group of enzymes, irreversibly releases a reporter molecule that can be detected. Starting from the earliest examples of colourimetric probes, increasingly sensitive and sophisticated substrates have been reported. In this review we present an overview of the recent advances in this field, covering an array of strategies including chromogenic and fluorogenic substrates, lanthanide complexes, gels and nanoparticles. The applications of these substrates for the detection of various glycosidases and the scope and limitations for each approach are discussed.

## 1. Introduction

The objective of this review is to provide a comprehensive overview of the enzyme activated, synthetic probes that have been developed for the detection of glycosidase enzymes. Glycosylation is one of the most important post-translational modifications of proteins and it is estimated that over 50% of human proteins are glycosylated.[1] Carbohydrates are often displayed in the form of glycoconjugates such as glycoproteins, proteoglycans and glycosphingolipids. They are ubiquitous in nature and play a pivotal role in a diverse range of biological processes including fertilisation, neuronal development, hormone activities and inflammatory responses.[2–4] Metabolism of glycans is therefore critical to the biological function of a given organism. Degradation of carbohydrates and glycoconjugates is challenging due to the stability of glycosidic linkages.[5] In nature, the hydrolysis of glycosidic linkages is catalysed by a

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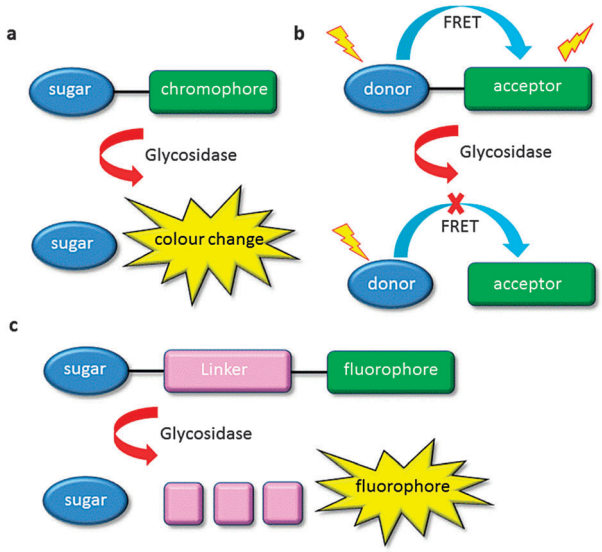
class of enzymes known as glycosidases. These enzymes increase the rate of hydrolysis by $10^{17}$ fold when compared to spontaneous hydrolysis events and exhibit exquisite substrate selectivity.[6] This remarkable acceleration in rate identifies glycosidases as highly efficient catalysts for this process. Despite the fact that all the glycosidase enzymes catalyse similar reactions, approximately 3% of the human genome is dedicated to the encoding of these enzymes.

The large number of glycosidases found in nature has led to their classification into various categories based on criteria such as catalytic mechanism, substrate specificity, site of glycosidic bond cleavage and amino acid sequence.[7,8] Glycosidases are referred to as *exo*, if they specifically act on the termini of polysaccharides, while *endo*-glycosidases promote internal hydrolysis of glycosidic linkages within a chain. The hydrolysis of the glycosidic bond can occur *via* one of two widely accepted mechanisms; proceeding with either retention or inversion of stereochemistry at the anomeric position, as outlined by Koshland in 1953.[9] In both cases, oxacarbenium ion intermediates and two amino acids (Asp or Glu) are involved in the mechanism. Several reports detailing the mechanism of action of the glycosidases on various carbohydrates have been published.[6,10,11] Glycosidases have been ordered into more than 120 different families based on amino acid sequence similarity and this classification has led to the generation of the CAZy database (available online at www.cazy.org).[12–14] Glycosidases grouped within a particular family share structural similarities and often their hydrolytic mechanisms are the same. Due to the complex and varying roles of glycans in biology, glycosidases have been associated with a number of important biological processes and applications.[15] Glycosidases have been reported to function as important gene markers, for example, the gene encoding βGal, *lacZ*, is extensively used as a reporter gene in animals and yeast.[16] A popular reporter gene in plants is the βGlu gene.[17] Glycosidase enzymes are also known to play a role in the pathology of several disease states. For example, aberrant glycosylation or glycosidase enzyme deficiencies are the marker for several pathological conditions including Gaucher and Parkinson's disease.[18–23] Xylanases and cellulases are widely exploited in industry. Of particular interest is the application of cellulases for the industrial production of bio-ethanol.[24] The realisation of glycosidases' dominant role in many biological and industrial processes has boosted research into the development of techniques to accurately detect and profile these enzymes so as to improve understanding of their function.




Scheme 1 Schematic illustration of (a) chromogenic-based reporters (b) distance-dependent FRET-based reporters (c) self-immolative activation of reporters; for glycosidase enzymes.

This review aims to survey the chemical tools developed to detect this important class of enzymes. The review will focus on chemical probes whereby a glycosidic linkage is hydrolysed and a reporter molecule is released. The review will not cover activity based glycosidase probes which have been comprehensively reviewed elsewhere.[25] The bioactivatable probes presented herein cover the early chromogenic glycoside substrates which are widely used commercially and the various fluorogenic substrates starting from the early coumarin structures up to more recent FRET-based systems. Lanthanides as tools for probing glycosidase enzymes are described with an emphasis on the development in the field of MRI. Other approaches for glycosidase detection such as incorporation of substrates into gels or nanoparticles are described highlighting the vast array of probes available, each differing in their mechanism of signal generation as outlined in Scheme 1.

## 2. Chromogenic substrates

The simplest and most well established method for the detection of glycosidases is based on the liberation of a coloured product upon enzyme catalysed hydrolysis of a glycosidic linkage in a suitable glycoconjugate. Many such glycoconjugates are commercially available and this method allows visualisation

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by the naked eye. This approach does not require the use of specialised equipment, and can be applied in glycosidase inhibitor high-throughput screening (HTS). In an effort to increase the intensity of released chromophore, Richardson and Perry have screened various colourimetric substrates for glycosidases.[26,27] Glycoconjugates of *p*-nitrophenol (*p*-NP) **1** were amongst the earliest chromogenic glycosidase substrates with the released nitrophenolate **2** displaying an intense yellow colour and absorbance at 405 nm (Fig. 1). The yellow colour of **2** is only visible at alkaline pH and its utility can be hampered when the absorbance of the inhibitor overlaps with that of **2**. However, this method is still widely used for the screening of glycosidase inhibitors and as a reference for the determination of absolute activities of enzyme samples. Wang and co-workers reported the novel amperometric detection of glycosidases based on *p*-NP glycosides.[28] The anodic signal arising from the subsequent electro oxidation of the released *p*-NP was determined to be directly proportional to the activity of the enzyme.

Indoxyl glycosides are powerful chromogenic substrates used in histochemistry, biochemistry and bacteriology.[29] Hydrolysis of the glycosidic bond releases a reactive indoxyl which is oxidised, under aerobic conditions, to produce an insoluble dimeric blue indigoid dye **4** (Fig. 1). The indoxyl group may be decorated with halides with the variation of the substitution patterns determining the colour.[30] The most commonly used substrate is 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal), **3** which is routinely applied to microbial cultures, however indigoid dyes tend to be insoluble in water thus requiring the addition of organic solvents which may partially inhibit enzyme activity.[29] The widespread utility of these dyes has been hampered by the challenging and low yielding synthesis of indoxyl glycoside derivatives, however recent reports by Thiem *et al.* have described improved synthetic approaches.[31,32] Maeda and co-workers have reported a novel assay using **3** as a substrate.[33] The $H_2O_2$ produced during the oxidation of the indoxyl reacts with residual **3** in the presence of horseradish peroxidase (HRP) to emit light, resulting in a novel chemiluminescent assay for detection of glycosidases. Colourimetric assays based on gold nanoparticles (AuNPs) have also been reported for the glycosidase enzymes (see Section 5.2). Furthermore, Ruan *et al.* reported that the formation of blue indigoid dye can be used for alkaline phosphatase enzyme assays using SERRS as a technique (see Section 5.3).[34]

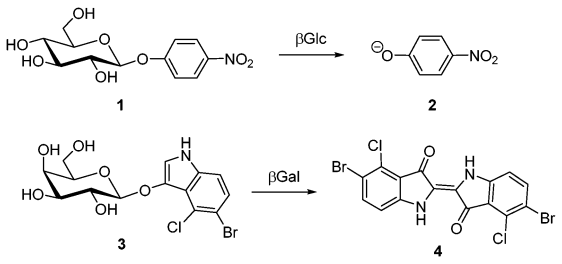


Fig. 1 Examples of chromogenic enzyme substrates.

# 3. Fluorogenic substrates

Fluorescence based measurements are several orders of magnitude more sensitive and tend to have lower limits of detection than colourimetric methods, thus can be used to detect enzymes at low concentration which is crucial for measurement in complex media. If carefully designed, fluorescent probes should exhibit negligible or no signal until they interact with their target enzyme allowing "on–off" switchability. To date, there has been a vast array of carbohydrate conjugated fluorophores reported with focus on umbelliferones, fluoresceins, and rhodamines, with each type having their own advantages depending on the particular application. Despite the wide array of fluorophores, fluorescent probes are rarely used in a clinical setting as conventional probes absorb in the UV/Vis region which is problematic since this is where biomolecule absorption is high. Although, in recent years the focus has shifted to fluorophores with large stokes shifts and significant bathochromic shifts to allow for *in vivo* studies.[35]

## 3.1 Coumarins

Coumarins are a class of lactones comprising of fused benzene and α-pyrone rings (benzopyrones). They are widely used, small-molecule fluorophores due to their high fluorescence intensity, good cell permeation, ease of preparation, and excellent solubility. The electron-rich π–π conjugated system imparts good charge-transport and fluorescent properties, rendering them excellent candidates for probing biologically-relevant species.[36,37] Coumarin-based fluorescent probes have been developed for the detection of magnesium,[38] zinc,[39] elastase,[40] thiophenols,[41] and kinase enzymes.[42] Dating back to the early 1950s, the coumarins of choice were the simple 4-methylumbelliferyl (4-MU) and resorufin derivatives, which were used as chemical probes for many different enzymes including esterases, phosphatases and glucosidases.[43–45] Furthermore, 6,7-dihydroxycoumarin serves as a colourimetric assay for βGlc due to enzymatic catalysed hydrolysis of the glycosidic linkage forming a brown-black complex in the presence of iron salts.[27]

In 1954, Mead and co-workers reported that glucuronides of umbelliferone were practically non-fluorescent whereas the corresponding hydrolysis products, hydroxycoumarins, were highly fluorescent at pH 10–11 and therefore could be used to determine βGlc activity.[46,47] Shortly thereafter, various derivatives of 4-MU were investigated as fluorogenic substrates for a range of glycosidase enzymes.[47–49] The identification of sialidases using an umbelliferyl probe **5** was reported by Warner in 1975 (Fig. 2).[50] This was followed by Schauer and co-workers[51] who reported a 4-trifluoromethylumbelliferyl derivative **6** which served as a very sensitive substrate for eight different sialidases, overcoming the limitations of **5** as the pH did not have to be adjusted to pH 10 to obtain full fluorescence (Fig. 2).

Mangan *et al.* reported a fluorometric cellulase assay based on a benzylidene protected β-cellotrioside, BzMUG3, using 4-MU as the reporter group. The assay is selective for the *endo*-1,4-β-glucanase with the rate of 4-MU formation being directly proportional to the enzyme activity.[52] Zheng and co-workers[53]

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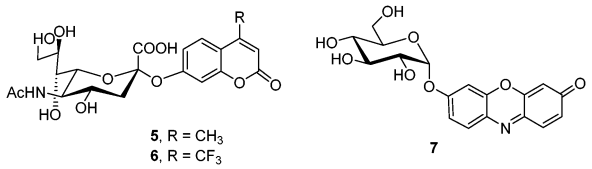


Fig. 2 Fluorogenic substrates based on the coumarin core structure.

reported resorufin-α-D-glucopyranoside **7** as a substrate for βGlc. This was superior to the previous assays developed which were based on the umbelliferone derivatives. This was followed by an αGal derivative (Fig. 2).[54] The emission of the product of the enzymatic reaction was red-shifted to 590 nm which reduced interference from background fluorescent compounds, as seen with 4-MU substrates.[55] Additionally, this allowed for continuous measurement of enzyme activity due to the lower p$K_a$ of the resorufin derivative, compared with 4-MU.

The use of coumarins for detection of glycosidases has been extended by Yang *et al.* who reported a novel platform **8** for the simultaneous measurement of both βGlc and phosphodiesterase activities, using 7-hydroxycoumarin as one of the fluorescent reporters (Fig. 3).[56] Notably, interactions of each distinct enzyme produce a different signal readout based on either intramolecular PET or FRET processes.

FRET is a widely used phenomenon in the field of molecular biology. It is a non-radiative process involving energy transfer from an excited state donor to a ground state acceptor. The efficiency of FRET is highly dependent on the spectral overlap integral, the relative orientation of transition dipoles, and proximity of the donor and acceptor to each other.[57–59] Extensive spectral overlap is displayed between coumarin emission and fluorescein excitation and this has been exploited for the design of FRET-based probes for enzymes.[60] FRET-based assays have been used for the detection of glycosyltransferase activity but glycosidase sensing using FRET remains underdeveloped, despite the widespread utility such a technique would possess.[61]

PET is a well-known phenomenon through which the fluorescence of a fluorophore is quenched by electron transfer from a donor moiety to an acceptor moiety.[62] PET systems traditionally exist in a 'fluorophore–spacer–receptor' format wherein electron transfer from the donor moiety quenches the acceptor in the "off" state. Binding of an analyte/enzymatic transformation restrains PET subsequently triggering an "on" state.[62,63]

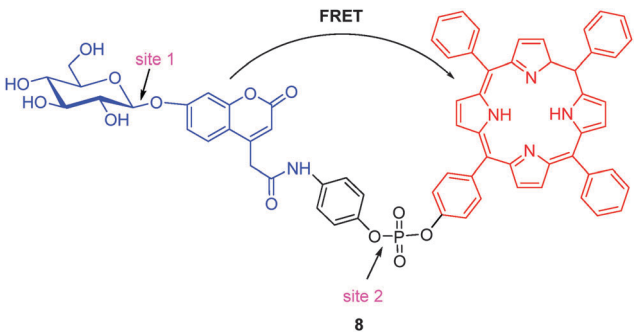


Fig. 3 FRET–PET system based on coumarin and TPP reporters showing dual sites for enzyme cleavage.[56]

The novel probe reported by Yang *et al.* possesses two enzyme cleavage sites and three separate fluorescent reporters with negligible cross-talk observed between them (Fig. 3).[56] The applicability of the probe to image enzyme activity in liver cell lines was determined using fluorescence microscopy. Fluorescent images of cell lines clearly showed the separate donor and acceptor components, with endogenous βGlc and phosphodiesterase activity producing blue and red fluorescence, respectively. Nagano utilised the spectral overlap of coumarin and fluorescein and a FRET-based approach to design a βGal labelling probe, CMFβGal, which can be monitored by changes in fluorescence.[64]

Withers and co-workers recently reported an elegant FRET probe using boron-dipyrromethane (BODIPY) and 7-hydroxycoumarin components to serve as the FRET acceptor and donor, respectively.[65] This probe allowed for the monitoring of three key hydrolytic enzymes involved in ganglioside degradation. Notably, the probe is the first example reported to detect and localise human neuraminidase activity in living cells.

Shin reported the use of cocktails of simple coumarin-conjugated compounds for the detection of glycosidase activity to generate activity profiles.[66] This was carried out using HPLC analysis and by examining the pattern of product formation in the enzymatic reactions, further illustrating the use of coumarin derivatives in rapid detection of enzymatic activity. Coumarins have also been reported to sensitize lanthanide ions in analyte-responsive probes reported by Borbas (see Section 4.1).[67,68]

### 3.2 Fluorescein

Fluorescein, along with rhodamine, belong to the superfamily of xanthene dyes. Fluorescein is a widely used fluorophore due to its good water solubility and its visible excitation and emission.[69,70] A widely exploited feature of fluorescein is the ability to switch between the non-fluorescent spirolactone and ring-opened forms.[69] Thus, fluorescein probes can be utilised which are non-fluorescent until reaction with a target molecule occurs.

Fluorescein di-β-D-galactopyranoside (FDG) **9** is a highly sensitive substrate for βGal and is commercially available (Fig. 4).[71–73] Substrate **9** is hydrolysed in a sequential manner to release fluorescein whose fluorescence can be monitored at 514 nm. The intermediate, fluorescein mono-β-D-galactopyranoside (FMG) **10**, although a good substrate for βGal, has a strong fluorescence and absorbance at the wavelength used for detection of fluorescein thereby hampering its utility.[74] Nolan *et al.* demonstrated that **9** could distinguish between *lac*Z+ and *lac*Z− cells using fluorescence-activated cell sorter (FACS) analysis.[72] MacDonald reported use of **9** in a multiwell plate system, demonstrating the higher sensitivity of **9** compared with colourimetric *o*-NPG and fluorogenic 4-MU, although it was noted that the turnover rate is slower due to the requirement for sequential glycosidic bond hydrolysis.[73] Moreover, fluorescein leakage from cells still remains a problem with **9** and the observed increase in fluorescence is known to be non-linear. Uttamchandani and co-workers have reported an α-*N*-acetylgalactosamine containing quenched activity-based probe (qABP). A FACS analysis approach was used in directed

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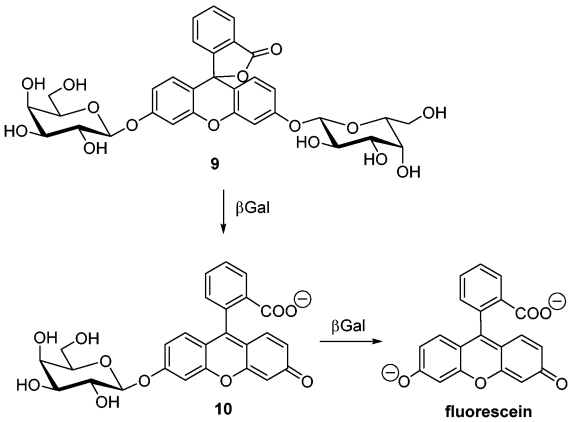


Fig. 4 Sequential FDG hydrolysis to yield fluorescein.

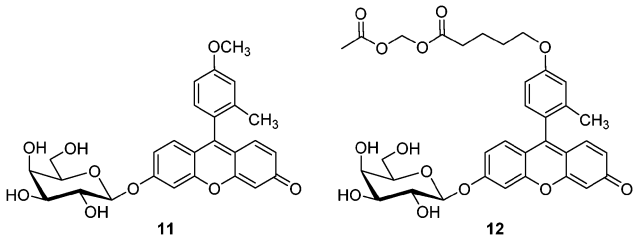


Fig. 5 Fluorescein derived PET substrates.

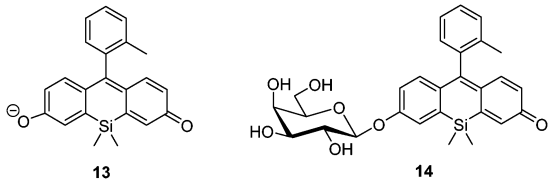


Fig. 6 Si-substituted fluorescein enzyme substrates developed by Nagano.

enzyme evolution experiments to identify an α-*N*-acetylgalactosaminidase variant with enhanced catalytic activity.[75]

Nagano demonstrated that the fluorescein molecule could be viewed as a donor–acceptor PET system, where the benzene and xanthene moieties within the fluorescein chromophore act as the donor and acceptor respectively.[62,76,77] Nagano substituted the carboxylic group of conventional fluorescein with a methyl moiety to generate a novel class of fluorescein molecules that displayed almost the same fluorescent properties and quantum yields as fluorescein, proving that the carboxylic group was not indispensable as previously reported.[78] They reported the enzyme-responsive TG-βGal (Tokyo Green) **11**, which was almost non-fluorescent with a small quantum yield until hydrolytic action of βGal which afforded a highly fluorescent moiety, due to PET being restrained, which existed mainly in the anionic form under neutral pH conditions (Fig. 5).[77] This probe has significant advantages over **9** in that it requires only a single hydrolytic step to release the fluorophore, thereby avoiding problems with moderately fluorescent **10**. In addition a linear response is observed between fluorescence intensity and βGal activity. Furthermore, **9** is well known to be membrane impermeable whereas the lower hydrophilicity of **11** imparts cell-permeability allowing for real-time imaging of living cells. Due to the fluorescent product of **11** being readily washed out of cells, it is unsuitable for *in vivo* imaging. Hence, Nagano subsequently reported AM-TG-βGal **12** which is based on **11** but demonstrated improved intracellular retention and was therefore suitable for *in vivo* tumour imaging (Fig. 5).[79] By introducing an acetoxymethyl ester, a dramatic fluorescence enhancement was observed upon addition of βGal and subsequent intracellular hydrolysis by esterase enzymes generated a fluorescent molecule with a free carboxylate group which is not readily leaked from cells.

Nagano has further demonstrated the utility of fluorescein for enzymatic detection by reporting a fluorescein analogue which fluoresces in the red region upon enzymatic hydrolysis.[80] By replacing the oxygen at the 10-position of the xanthene unit in fluorescein with a Si atom, it was observed that deprotonation of the resulting phenolic substrate furnished a product 2Me-TM **13** that red-shifted the absorbance by 110 nm relative to the protonated form. This red shift is due to the difference in σ*–π* conjugation between neutral and anionic forms. The related glycosylated substrate 2Me-TM β-Gal **14** was prepared (Fig. 6).[80–82] Upon exposure of **14** to βGal, the expected dramatic red shift in the absorbance was observed and was accompanied by an increase in fluorescence intensity. A large fluorescence increase was also seen when **14** was incubated with *lac*Z+ cells, when excited at 580 nm. This strategy of incorporating Si into xanthene dyes and the biological applicability has recently been reviewed by Nagano.[83]

### 3.3 Rhodamine

Rhodamine dyes have been one of the platform molecules for the design of fluorescent probes due to their high extinction coefficients, quantum yield, and photostability.[84] In acidic media, rhodamine exists in a cationic form due to the carbonyl being protonated while a zwitterionic form is observed in basic solution due to dissociation. This negative charge induces a hypsochromic shift in the absorbance and fluorescence along with a reduction in extinction coefficient due to the inductive effect on the central carbon of the xanthene moiety.[84] Analogous to fluoresceins, rhodamines are non-fluorescent when in the spirolactone form and strongly fluorescent when in a ring opened form. Furthermore, a key feature of rhodamines is that their fluorescence capabilities can be controlled by PET.[69,84,85]

In 2011, Nagano identified a rhodol derivative capable of serving as a probe for various hydrolases.[86] Rhodol, being a hybrid of fluorescein and rhodamine, exhibits the desirable characteristics of rhodamine and fluorescein but displays less pH-dependence making it an ideal candidate fluorophore.[87,88] Moreover, as demonstrated by Nagano, conversion of the rhodamine carboxylate to a hydroxymethyl group induces spirocyclization and suppression of background fluorescence in an *O*-alkylated rhodol. Thus, due to the presence of a phenolic hydroxyl group in rhodol, it was used as a βGal probe, HMDER-βGal, **15** which is masked with a glycoside until enzyme activation triggers liberation of HMDER **16** and concomitant fluorescence enhancement occurs

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(Fig. 7). A 76-fold increase in fluorescence was observed upon addition of the enzyme and a clear *in situ* visualisation and co-localisation of **15** occurred with βGal activity in *Drosophila melanogaster*. Furthermore, **15** displayed a log*P* and log*D* one unit larger than the previously reported fluorescein-based **11** suggesting it has improved cell permeability and *in vitro* applicability.[77]

Classic rhodamine dyes exhibit absorption and emission in the visible range, however, pioneering work by Nagano has led to the emergence of biologically applicable near-infrared (NIR) rhodamine dyes. It was reported that replacement of the oxygen atom in the xanthene ring with group 14 elements improves the fluorescent properties of classic rhodamines. A dramatic red-shift to NIR emission was observed due to the relatively low lying LUMO levels of the group 14 elements allowing the fluorescent properties to be readily controlled.[89–91]

Recent studies have been carried out by both Urano and Nagano in the area of enzyme-activated photosensitizers. The Nagano group reported βGal activated photosensitizers based on thiazole orange, PhoTo-Gal, and fluorescein, TGI-βGal.[92,93] However, these aforementioned probes were unable to induce targeted cell death. Replacing the oxygen atom at the 10-position of the xanthene moiety with a Se atom creates a more effective photosensitizer, as reported by Detty *et al.*[94] Thus, recently published work by both Urano and Nagano demonstrated the selective ablation of β-galactosidase-expressing cells by a Se-substituted rhodol derivative, HMDESeR-βGal **17**, based on their previously reported **15** (Fig. 7).[95,96] Incorporation of a galactosyl moiety into **18** suppresses phototoxicity, due to the presence of predominant spirocyclic xanthene form. Selective, enzyme-catalysed, glycosidic bond cleavage liberates phototoxic **18** which has the ability to produce $^1O_2$ upon irradiation at 532 nm. Furthermore, *ex vivo* analysis of *Drosophila* showed that **18** induced cell death specifically in β-galactoside-expressing regions.

The Nagano group reported a series of hydroxymethyl rhodamine derivatives capable of detecting and visualising protease and glycosidase activity, on the basis of the spirocyclization capabilities of rhodamine.[97] They found that the acetylated derivative of HMRG **19** existed in the closed form and was non-fluorescent due to loss of π-conjugation compared with phenolic **19** which was highly fluorescent and existed in the open form. Based on this observation, they incorporated enzyme specific substrate moieties in place of the acetyl group to form enzyme activated, fluorescent probes with "on–off" switchability. A galactose moiety was coupled to **19** *via* a carbamate linker to give βGal-HMRG **20** which exhibited no fluorescence until enzymatic action triggered the formation of the open form **19** with concomitant increase in fluorescence intensity (Fig. 7). Compound **20** was found to exhibit less background signal and improved fluorescence intensity, compared to the previously reported **15**.[96] Furthermore, **20** was also capable of visualising βGal activity in living cells.[97]

### 3.4 Near-infrared emitting fluorophores

NIR fluorophores have been the subject of intense research and development, but despite this, only a limited number of enzymatically-active NIR probes have been developed. The use of conventional fluorophores for *in vivo* applications is problematic due to high absorption of biomolecules in the UV/Vis region, easy scattering of visible light, and autofluorescence resulting in a low signal-to-noise ratio.[35] Conversely, light in the NIR region is more suitable for *in vivo* applications as this represents the window where autofluorescence is at a minimum resulting in a higher signal-to-noise ratio and deeper penetration into tissues. Extension of the π–π conjugation system of conventional dyes is a common strategy for obtaining NIR fluorophores. Work by Renard and Hahn highlighted strategies for converting simple common dyes into water-soluble, photostable, far-red enzyme-activated fluorophores.[98,99] Furthermore, a suitable *in vivo* probe must be endowed with a pharmacokinetic profile which allows it access to the native enzymes in order to provide a detectable signal.[35,100,101]

Weissleder and co-workers reported a far-red fluorescent probe for βGal, DDAOG **21**, which displays a dramatic bathochromic shift of 50 nm upon hydrolysis to DDAO permitting product detection even in the presence of intact substrate **21** and more efficient tissue penetration (Fig. 8).[102] The excitation and emission spectrums of hydrolysis product DDAO overlap to a large extent hampering its utility; nevertheless, the substrate was successfully employed for imaging of βGal expression *in vivo*. Gong and co-workers developed a protocol for the use of **21** in cultured cells highlighting the 12-fold higher signal-to-background ratio of **21** compared to *o*-PNG.[103] Compound **21** is commercially available and widely used in industry due to the

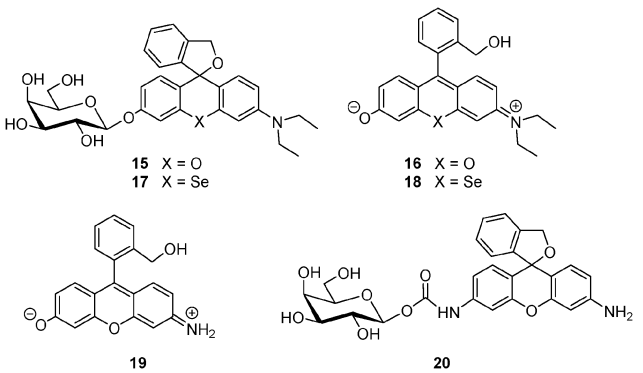


Fig. 7 Glycosidase activated rhodamine substrates.

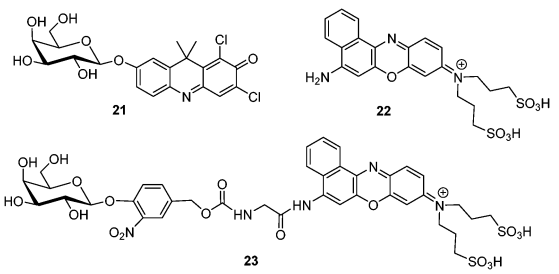


Fig. 8 NIR-based glycosidase substrate fluorophores.

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relatively low p$K_a$ of hydrolysis product, DDAO, allowing continuous monitoring of enzyme activity at physiological pH.

Weissleder and Tung reported a new water-soluble, far-red fluorogenic molecule, 2SBPO **22** (Fig. 8). It was found that masking of the primary amino group of **22** resulted in a weak fluorescence and absorbance, which upon proteolytic cleavage generated a strong bathochromic fluorescence with a modest stokes shift.[104,105] This observation was later used to generate a substrate for βGal detection, Gal-2SBPO **23** (Fig. 8).[106] Although βGal substrates are restricted to hydroxyl-linked fluorochromes, **22** was incorporated through a *p*-benzyloxycarbonyl moiety which is liberated *via* a self-immolative mechanism. Following enzymatic cleavage, **23** is hydrolysed to liberate **22** which can be followed by absorbance and fluorescence at 630 nm and 670 nm, respectively. Additionally, the reaction can be followed by the liberation of 4-hydroxy-3-nitrobenzyl alcohol due to a yellow to blue colour change observed upon hydrolysis.

Recently, Tung and co-workers have developed a new NIR cyanine-based probe for detection and imaging of βGal, based on the previously reported fluorophore QCy7 **24** (Fig. 9).[107,108] GQCy7 **25** has a structure similar to other cyanine dyes and functions by masking the phenol functional group and disrupting the π-electron system of the dye rendering it "off". Upon enzyme action, subsequent self-immolative elimination restores the conjugation of the system resulting in a 110-fold fluorescence increase of **24** emitting at 680 nm. Compound **25** exhibited poor membrane permeability thus can only be used in cell-free assays. Conversely, the acetylated carbohydrate derivative, AcGQCy7, was found to be cell-permeable and was effectively retained in cells, thus proving suitable for βGal imaging. The mechanism of intracellular fragmentation involves a thiol-dependent Michael addition followed by retro-Knoevenagel condensation to form a thionium ion which is subsequently hydrolysed by intracellular esterases and βGal to release fluorophore **26**. However, **26** exhibits a dramatic hypsochromic shift to 560 nm which diminishes the potential application of AcGQCy7 for *in vivo* studies.

Squarine dyes exhibit desirable, long wavelength absorption and high extinction coefficients rendering them useful candidates for NIR applications.[109] Nagano and co-workers reported a novel design strategy for NIR probes based on a squarylium scaffold 6SqGal **27** to visualise βGal activity (Fig. 10).[110] Based on the fact that polymethine dyes exhibit fluorescence enhancement and a dramatic red shift in their emission upon interaction with serum proteins, it was proposed that the introduction of an enzyme-active functional group into the dye could suppress these interactions. Selective cleavage of the glycosidic bond triggers a dye–protein interaction accompanied by a fluorescence enhancement.[110–112] The squarylium hydrolysis product of **27**, 6SqOH, displayed a significant spectral change upon enzymatic action and subsequent protein interaction. Moreover, **27** exhibited a binding parameter of two orders of magnitude lower than hydrolysis product 6SqOH confirming that the glycan moiety suppresses the protein–dye binding affinity. Bright fluorescent images and notable subcellular localisation of **27** in *lacZ*+ cells was observed. Furthermore, the ability of **27** to image βGal activity *in vivo* in mice was demonstrated with the enhancement of fluorescence intensity of a targeted βGal-encoding liver plasmid clearly visible.

Vocadlo and co-workers reported a glucocerebrosidase (GCase) substrate **28** which is a fluorescence-quenched substrate before enzyme action (Fig. 11).[113] Substrate **28** showed localisation within the lysosome and allowed the time-dependent monitoring of endogenous GCase activity within human fibroblasts. Substrate **28** contains the quencher group Black Hole Quencher 2 (BHQ2) attached to the anomeric center using a spacer and the fluorophore BODIPY attached to the 6-position as modification at this position has been reported to be tolerated.[114] When intact, **28** is effectively quenched but upon hydrolytic cleavage NIR fluorescence is observed. Compound **28** shows excitation and emission at 574 nm and 595 nm, respectively, and an impressive 99.9% quenching efficiency producing a probe with good dark-to-light switchability. Furthermore, live cell imaging in human fibroblasts was

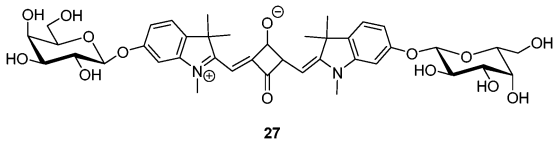


Fig. 10 Squarine-based 6SqGal **27**.

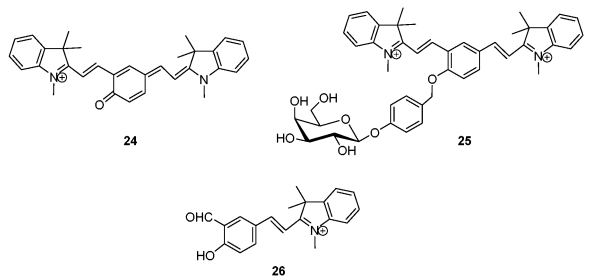


Fig. 9 Self-immolative NIR glycosidase substrates.

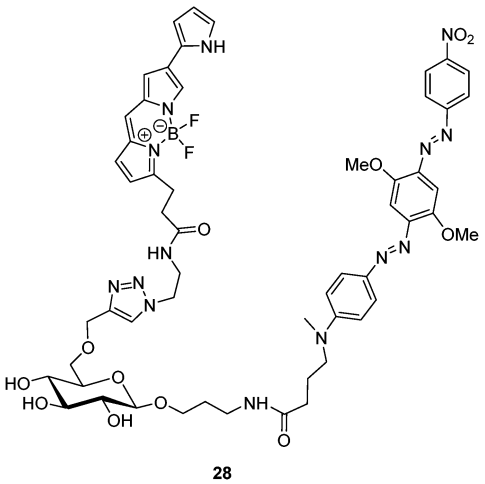


Fig. 11 Fluorescence quenched GCase substrate.

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demonstrated and the fluorescence increase proved to be time and dose-dependent while exhibiting no cytotoxicity.

## 4. Lanthanide ions

The intricate optical and spectroscopic properties of the lanthanides ions make them useful spectroscopic tools for probing biological processes. The lanthanides exhibit sharp, narrow bands in their emission spectra and a large pseudo Stokes shift.[115] Perhaps the most useful property of the Ln(III) ions is their long-lived excited states, in the millisecond range, owing to the parity-forbidden f–f transitions.[116,117] This allows for the implementation of time-resolved luminescence measurements where there is a delay between the excitation and measurement of emission, obviating problems with autofluorescence.[118,119] Due to the Laporte forbidden nature of the f–f transitions, the Ln(III) ions have inherently low molar absorption coefficients making direct excitation of the Ln(III) problematic. This is overcome by the incorporation of an appropriate chromophore into the Ln(III) complex to allow "indirect sensitisation" of the Ln(III) ion. Furthermore, the high coordination requirements of the Ln(III) ions must be fulfilled due to their inherent toxicity.[118,120,121]

One of the most commonly used ligand frameworks for lanthanide complexation is cyclen (1,4,7,10-tetraazacyclododecane).[122] Structurally diverse complexes based on the cyclen framework can be designed and constructed through the alteration of the pendant arms resulting in an architecture endowed with high kinetic and thermodynamic stability. Given their potential utility, there has been a recent surge of interest in the chemistry of the lanthanides. A huge array of diverse applications have been reported including their role in the formation of Langmuir–Blodgett films,[123,124] in the sensing of biologically relevant anions and metal ions,[125–130] and in the determination of 3D protein–ligand complexes due to their prominent use as shift reagents in NMR spectroscopy.[131,132] Moreover, luminescence measurements represent a facile, non-invasive method to monitor enzymatic reactions.[133,134]

### 4.1 Probes based on lanthanide luminescence

Borbas and co-workers reported a series of novel analyte-responsive luminescent probe capable of detecting $H_2O_2$, $Pd^{0/2+}$, $F^-$ and βGal by alternating the analyte-responsive moiety.[67] They previously demonstrated that coumarins sensitise Tb(III) and Eu(III) ions efficiently and applied this concept to develop a non-sensitising precursor **29**, based on a Ln(III)–cyclen framework that undergoes a chemoselective reaction upon analyte activation to generate a coumarin sensitizer **30** (Fig. 12).[135] Additionally, it was shown that βGal activity could be monitored in cells expressing *lacZ+* in real-time, resulting in intense Eu(III)-centred emission.

This antenna-triggered sensitisation strategy was later elegantly employed by Borbas to produce a multiple analyte detection system.[68] A method was developed using varying lanthanide-based probes with different emission wavelengths that allowed a multi-colour detection method. The activity of βGal and alkaline phosphatase was simultaneously monitored

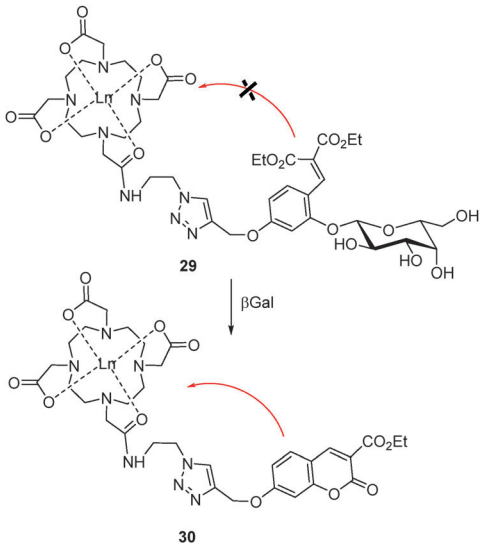


Fig. 12 Glycosidase triggered lanthanide antennae formation.

following emission at 545 nm and 655 nm for Tb(III) and Eu(III), respectively, with no spectral cross-talk observed between the emitters. Furthermore, the feasibility of a three-colour detection system was demonstrated using a coumarin-based organic fluorophore, whose absorption and emission is outside the range of the two lanthanide emitters. Thus, three non-interacting analytes; βGal, alkaline phosphatase, and $H_2O_2$ were monitored at 450, 545, and 655 nm, respectively.

### 4.2 Probes based on lanthanide relaxivity

The work of enzyme-responsive MRI contrast agents was pioneered by Meade and co-workers, with the development of the first bio-responsive *q*-modulated contrast agent, Egad **31** (Fig. 13).[136] NMR relaxivity of the galactose-substituted Gd(III)–cyclen complex was selectively "switched-on" by removal of the galactopyranoside with βGal. Exposure to βGal causes the transition from a weak to a strong relaxivity state by affecting the ability of galactopyranose to modulate the rate of water exchange at the Gd(III) centre. This was confirmed through recording the exponential decay of luminescence of a Tb(III) derivative to determine the hydration number, *q*, which confirmed a 40% increase in the number of inner sphere water

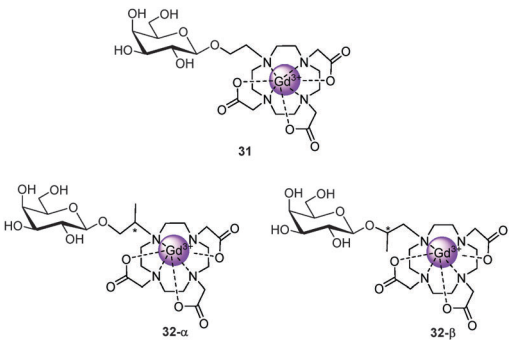


Fig. 13 "Smart" enzyme-responsive MRI contrast agents.

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molecules, upon hydrolysis of the glycosidic bond. Additionally, this change could be visualised in an MR image due to the 20% decrease in longitudinal relaxation time ($T_1$) values accompanying enzymatic hydrolysis.

Complex **31** was further developed to improve its low relaxivity to allow for adequate *in vivo* imaging. The rigidity of **31** was increased through the introduction of an α-methyl group limiting water access to the Gd(III) ion, generating EGadMe **32**-α (Fig. 13).[137] This modification caused a relaxivity change of 3-fold upon enzymatic cleavage thus improving on the previously developed **31** and paved the way from more robust *in vivo* MRI imaging agents. Further mechanistic investigations were reported which demonstrated that varying substitutions patterns on the linker between the galactopyranose and cyclen had a profound effect on the MR image produced.[138] Structural isomers of EGadMe **32**-α and the mechanism for enzyme activation in both the presence and absence of carbonate was explored. It was determined that the position of the methyl group (**32**-α or **32**-β) had a significant impact on the mechanism of water exclusion prior to enzymatic cleavage with the stereochemistry of the methyl group appearing to play no significant role. Substitution at the α-position in **32**-α allows for only outer-sphere contributions with the galactopyranose unit blocking water access whereas **32**-β displays dependence on carbonate concentration, suggesting that the sugar is not obstructing water access to the Gd(III) ion.

The enzyme kinetics of **32**-α were not optimal, so in order to improve on this, Meade designed a new enzyme-activated agent **33** which operated *via* a self-immolative mechanism activated by βGlc to furnish **34** (Fig. 14).[139] The kinetics was quantified by the release of 4-hydroxy-3-nitrobenzyl alcohol ($\lambda_{max}$ = 422 nm). Complex **33** proved to be a better βGlc substrate than the standard assay substrate, *p*-nitrophenyl-β-D-glucuronide.

Since the initial reports, detection of βGal through "smart" MR contrast agents has been pursued by several groups. Nagano developed a receptor-induced magnetisation enhancement (RIME)-based βGal activated MRI contrast agent, **35** whereby increase in longitudinal water proton relaxivity ($r_1$) occurs upon binding to a macromolecule, due to the slowing of molecular rotation (Fig. 15).[140] Cleavage of the glycosidic bond

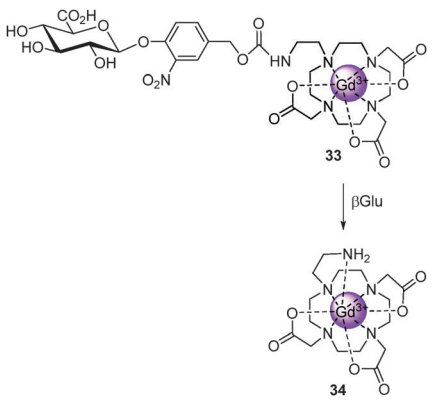


Fig. 14 Self-immolative βGlu MRI contrast agent.

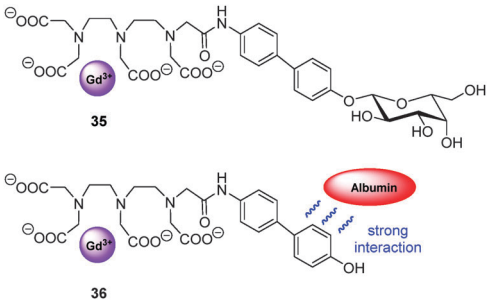


Fig. 15 RIME-based βGal activated MRI contrast agent.

of **35** releases a more hydrophobic aryl group **36**, whose affinity for human serum albumin (HSA) is increased through a non-covalent interaction resulting in slower molecular tumbling and a strong relaxivity state.

Wang and Cheng reported a self-immolative Gd(III) contrast agent, Gd(DOTA-FPG) **37** whereby cleavage of the glycosidic bond by βGal caused an electron cascade, resulting in release of an electrophile which subsequently reacts with HSA to form a covalent adduct **38** (Fig. 16).[141] A significant decrease in $T_1$ was observed in the presence of βGal and HSA. Furthermore, the signal intensity was selectively increased in tumours with βGal gene expression *in vivo*. However, it should be noted that HSA tends to reside inside extracellular compartments while βGal is found in the cytoplasm which hampers the utility of methods reported by Wang and Nagano. Engelmann *et al.* tackled the problem of cell-permeation of MRI probes through the conjugation of peptide D-Tat$_{49-57}$, in order to generate a cell-permeable, bimodal βGal targeting agent.[142] This peptide-conjugated Gd(III)-DOTA complex is only selectively cleaved and activated in βGal-containing cells.

In an effort to amplify the MRI response at targeted cells, Aime *et al.* published a Gd(III)-DOTA reporter which contained on its surface a βGal-tyrosine moiety, which was capable of reporting on βGal expression in melanoma cells.[143] Accumulation of a metal complex inside cells by the formation of oligopolymers had previously been reported by Bogdanov *et al.*[144,145] Aime reported that following the action of βGal and tyrosinase, melanin oligopolymers were formed accompanied by an increase in water proton relaxation rate and therefore a good relaxation enhancement effect. Also noteworthy, is the use of paraCEST (paramagnetic chemical exchange saturation transfer) for detecting enzymatic activity. The use of paraCEST was illustrated by Toth *et al.* whereby action of βGal converted a masked carbamate to an

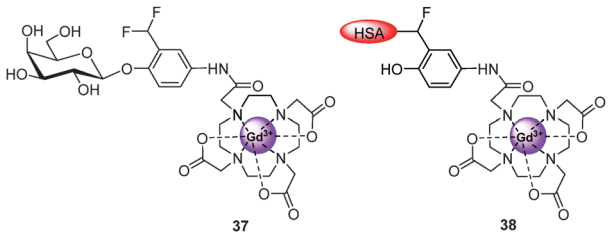


Fig. 16 βGal MRI contrast agent.

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amine with concomitant changes in the paraCEST properties of a Yb(III)-chelating unit due to the different exchange rates and chemical shifts of the carbamate and amine protons.[146] This approach allowed the monitoring of the kinetics of enzyme catalysed bond cleavage.

# 5. Other approaches

## 5.1 Gels

The applications of biomaterials for sensing of enzyme activity using "smart", stimuli-responsive materials and synthetic supramolecular pores has been reported for various enzymes.[147,148] Gels contain a large amount of immobilized solvent and are therefore regarded as an intermediate state between a dry and wet system. Hydrogels typically consist of chemically or physically cross-linked polymers and are heavily investigated for application as "soft" materials.[149–151] Hamachi and co-workers reported a novel hydrogelator comprised of a glycosylated amino acid ester scaffold which spontaneously formed a gel and exhibited unique amphilicity. This semi-wet structure allows for an aqueous medium for the inclusion of native-state enzymes whilst having hydrophobic domains for monitoring the enzymatic transformations, allowing a convenient sensing domain to monitor glycosidases and various other hydrolytic enzymes.[149,152,153] Subsequently, Hamachi reported a colourimetric hydrogel which showed a distinguishable naked eye colour change in response to the presence glycosidases.[154] Unique, bola-amphilic glycolipids **39** self-assembled to form a supramolecular hydrogel which exhibited a yellow-to-orange colour change along with gel-to-sol transition when subjected to βGlc, allowing for rapid detection of the enzyme (Fig. 17).

Maitra *et al.* reported a novel sensing of enzymes using a gel-based Tb(III) luminescence assay, obviating the need for chelation of the lanthanide ion by a multidentate ligand.[155] Previous studies showed that 2,3-dihydroxynaphthalene (DHN) sensitization of lanthanides could be achieved using a rigid cholate gel matrix.[156] This method was extended to produce "pro-sensitizer" **40** as a βGlc substrate, which, upon action of an enzyme causes luminescence of the gel (Fig. 17).[155] This principle was then later applied to generate functionalised vesicles by embedding Tb(III)-cholate aggregates into the membrane of unimellar 1,2-dioleoyl-*sn-glycero*-3-phosphatidylcholine (DOPC) vesicles which act as luminescent probes for the enzymatic transformations of lipase and βGlc.[157]

## 5.2 Nanoparticles

Surface plasmon resonance (SPR) spectroscopy is a sensitive technique which is increasingly being used for sensor technology.

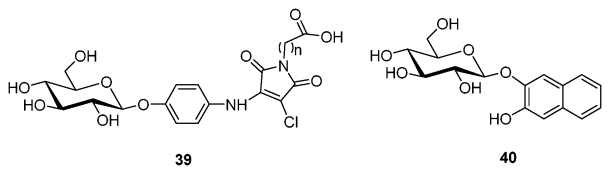


Fig. 17 Gel incorporated glycosidase substrates.

Gold nanoparticles (AuNPs) have inherent sensing abilities due to their interesting optical and electronic properties and they have served as a versatile platform for exploring many facets of sensor technology. AuNP-based colourimetric assays have been developed which probe the interactions between a recognition unit immobilized on the AuNP and an analyte.[158–160] Many of these colourimetric assays rely on the ability of AuNPs to aggregate and the subsequent shift of the SPR band to higher wavelengths resulting in a visible colour change.[161,162]

Zhang and co-workers reported a colourimetric method for the detection of αGlc activity and inhibitor screening based on AuNPs.[163] The method is based on the specific recognition between 1,4-phenylene diboronic acid (PDBA) and 4-aminophenyl-α-D-glucopyranoside (pAPG). Addition of the PDBA to the pAPG triggers extensive agglomeration of the AuNPs and colour change from red to blue with concomitant appearance of a new absorbance peak at 650 nm due to the interaction between the boronic acid and the glycan moiety. However, in the presence of αGlc, no aggregation or colour change is observed due to the cleavage of the glycosidic bond and the absence of binding interactions between PDBA and the free phenol group. This method was used to screen the activity of the well-known glucosidase inhibitors. A self-immolative approach was adopted by Kikuchi *et al.* who reported a AuNP assay for the detection of βGal and βGlc and its application for glycosidase inhibitor screening (Fig. 18).[164] They synthesised glycoside-masked lipoic acid derivatives **41** which undergo self-immolative elimination to produce primary amines upon cleavage of the glycosidic bond. Under physiological conditions, the charged ammonium groups electrostatically interact with neighbouring lipoate groups on neighbouring AuNPs and a successive shift in the SPR from 521 nm to 700 nm associated with AuNP aggregation was observed, accompanied by a colour change clearly visible to the naked eye. Furthermore, they demonstrated the utility of such a system by screening the well-known glycosidase inhibitors, D-galactal and castanospermine.

## 5.3 Surface enhanced resonance Raman scattering (SERRS)

SERRS is an alternative approach to fluorescence and is becoming a widely used methodology for bioanalysis. It produces characteristic bands and detailed fingerprint of the target without the need for tedious separations and can be carried out in aqueous solutions due to the weak scattering from water molecules. It is a sensitive technique allowing the detection of single molecules, overcoming the insensitivity associated with Raman spectroscopy.[34,165,166] "Masked" enzyme substrates that are initially undetectable by SERRS until action by a hydrolytic enzyme allows for information-rich vibrational spectra to be produced which lend the technique favourably to identifying multiple components in a mixture.[166]

Moore *et al.* demonstrated that 8-hydroxy quinolinyl azo dye derivatives can serve as SERRS reporters for the detection of hydrolytic enzymes.[167,168] They used a SERRS approach to simultaneously detect alkaline phosphatase substrate **42** and βGal substrate **43** by masking the 8-hydroxy group with an enzyme hydrolysable moiety reducing the surface enhancement of the Raman response (Fig. 19). The SERRS signal is then

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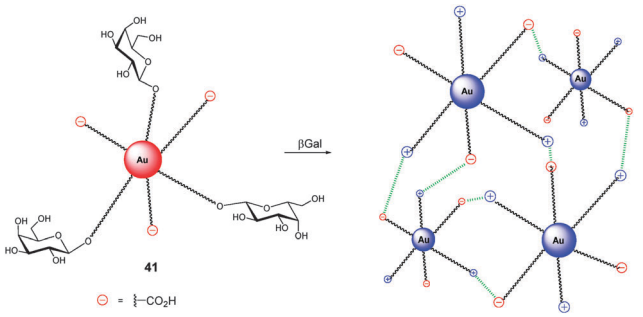


Fig. 18 AuNP glycosidase reporters.

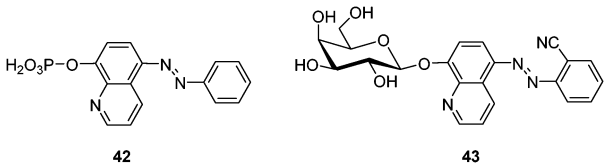


Fig. 19 Glycosidase SERRS reporters.

switched "on" following enzyme action with the emergence of each dye displaying distinct Raman bands allowing them to be differentiated.

## 6. Conclusions

The above account has clearly demonstrated the diversity of chemical substrates that have been developed for the detection of glycosidase enzymes. Although significant progress has been made, particularly in the field of NIR fluorophore development and application, there still remain several challenges to overcome prior to the emergence of a general strategy for glycosidase detection. As outlined in the introduction, due to the critical biological role of glycosidase enzymes in nature, there exists an exciting opportunity to develop probes suitable as tools for medical diagnostics and drug screening. While some preliminary examples of *in vivo* assays have been described above, significant further development is required in order to develop tools suitable for probing complex glycobiology processes. The ability to assay multiple glycosidase enzymes simultaneously using the same substrate coupled with advances in sensitivity for the detection of reporter molecules represents a major advance in the field. The development of efficient strategies to probe *endo*-glycosidases, without recourse to the use of multiple enzymes remains a significant challenge for the field. It is anticipated that the development of novel strategies for the detection of glycosidase enzymes will remain an active area of research in the future.

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